

## Segregation of hematuria in thin basement membrane disease with haplotypes at the loci for Alport syndrome

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### Segregation of hematuria in thin basement membrane disease with haplotypes at the loci for Alport syndrome.

**Background.** Inherited hematuria is common and is usually attributed to thin basement membrane disease (TBMD). The aim of this study was to determine how often hematuria in families with TBMD segregated with haplotypes at the chromosomal loci for autosomal recessive and X-linked Alport syndrome (COL4A3/COL4A4 and COL4A5, respectively).

**Methods.** The families of 22 individuals with TBMD on renal biopsy and with urinary glomerular red blood cell (RBC) counts of more than 50,000/mL were studied using phase-contrast microscopy of the urine and DNA microsatellite markers. Eighteen families had at least two members with hematuria.

**Results.** Hematuria segregated with or was consistent with segregation at the COL4A3/COL4A4 locus in eight (36%) families ( $P < 0.05$  in 5 of these) and at the COL4A5 locus in four (18%) families ( $P < 0.05$  in 2). The lack of segregation in the other 10 (45%) families may have occurred because of incomplete penetrance of the hematuria, de novo mutations, coincidental hematuria in other family members, or the presence of a novel gene locus. In four different families, three of which had hematuria that segregated with the COL4A3/COL4A4 locus, four family members with the hematuria haplotype had spouses with coincidental hematuria (4 of 29, 14%). However, none of their four offspring who had also inherited the hematuria haplotype had the clinical features of autosomal recessive Alport syndrome.

**Conclusions.** Hematuria in families with TBMD commonly segregates with the COL4A3/COL4A4 locus and thus results from mutations in the same genes as autosomal recessive Alport syndrome. Sometimes TBMD may be confused with the carrier state for X-linked Alport syndrome. However, nearly half of the families in this study had hematuria that did not segregate with the loci for either autosomal recessive or X-linked Alport syndrome.

Thin basement membrane disease (TBMD) or “benign familial hematuria” probably affects at least 1% of the community and is thus the most common renal condition

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after infections, hypertension, and stones [1–3]. Individuals with TBMD have persistent microscopic glomerular hematuria, sometimes with proteinuria or hypertension, but renal function is usually normal [1–5]. The diagnosis is made when there is a thinned glomerular basement membrane (GBM) on ultrastructural examination of the renal biopsy. TBMD is often inherited, when it then demonstrates autosomal dominant transmission and affects approximately half of successive generations [2–4].

It has recently been suggested that TBMD represents a carrier state for autosomal recessive or X-linked Alport syndrome [6]. The evidence for this is that heterozygous carriers of the causative mutations in autosomal recessive Alport syndrome [7, 8] and female carriers of X-linked Alport syndrome [9] can have a thinned GBM that is identical to that seen in TBMD.

The aim of this study was to determine how often hematuria in families with TBMD segregated with the genetic loci for autosomal recessive and X-linked Alport syndrome. Type IV collagen is the predominant protein found in the GBM, and autosomal recessive and X-linked Alport syndrome affect the genes encoding the  $\alpha 3(\text{IV})$ ,  $\alpha 4(\text{IV})$  (COL4A3/COL4A4), and  $\alpha 5(\text{IV})$  (COL4A5) collagen chains, respectively [10, 11].

### METHODS

Index cases were consecutive patients with TBMD referred because of a family history of hematuria ( $N = 10$ ) or, subsequently, consecutive patients diagnosed with TBMD at our medical center ( $N = 12$ ). Only one family that was invited refused to take part in the study.

All index cases had TBMD on renal biopsy and more than 50,000 glomerular or dysmorphic red blood cells (RBCs)/mL on phase-contrast microscopy [12] of a mid-stream urine specimen on two or more occasions. These urines contained at least three different populations of RBCs. TBMD was diagnosed when there was diffuse thinning of the GBM with an average width  $<250$  nm in adults [5] or  $<200$  nm in children [13] using 50 mea-

surements of the GBM where it intersected a grid overlying the electron micrograph [14]. None of the index cases had light microscopic or immunofluorescent features of other forms of glomerulonephritis in addition to TBMD. In each family, the eyes of the index case and often other family members were examined by an experienced ophthalmologist for lenticonus or retinopathy consistent with autosomal recessive or X-linked Alport syndrome, and a hearing questionnaire was administered. No abnormalities were detected. Furthermore, no one at the time of entry to the study knew of a family member with Alport syndrome, renal failure, or inherited hearing loss.

All available family members were examined. Hematuria was used to identify affected individuals, and "affected" individuals had  $\geq 30,000$  glomerular RBC/mL urine.

DNA from peripheral blood leukocytes or buccal smears was extracted and amplified using standard techniques and published conditions. Linkage to the COL4A3/COL4A4 locus was examined using at least three of the following, CA11, D2S351, D2S401, PAX3 microsatellites and a COL4A4 HaeIII intragenic restriction fragment length polymorphism (RFLP), and linkage to the COL4A5 locus was examined using at least three of the following, 2B6, DXS456, 2B20, DXS1105, and DXS1120 microsatellite markers. Each 10  $\mu$ L reaction mix contained 50 ng genomic DNA, 100 ng primers specific for the microsatellite markers (with the 3' primer end-labeled with  $\gamma^{32}$ P-ATP using T<sub>4</sub> polynucleotide kinase; Geneworks, Adelaide, Australia), 1 mmol/L dNTP (Pharmacia), 1  $\mu$ L of 10  $\times$  reaction buffer, 2.5 mmol/L MgCl<sub>2</sub>, and 0.5  $\mu$  Taq polymerase (Geneworks).

After amplification, 2  $\mu$ L of formamide stop solution [95% formamide, 20 mmol/L ethylenediaminetetraacetic acid (EDTA)] were added to each reaction tube, which was then incubated at 95°C for 30 minutes and chilled. Six microliters from each reaction mix were loaded into individual wells and electrophoresed in a 7% denaturing polyacrylamide gel at 50°C for two to four hours. Alleles were determined by exposing the gel to x-ray film (Biomax; Kodak, Rochester, NY, USA) for one to three hours and assigned numbers according to designated band size. Genotypes for the COL4A4 RFLPs were determined after amplification, restriction with HaeIII (Boehringer Mannheim, Mannheim, Germany) at 37°C for four hours, and electrophoresis in a 1% agarose gel. Haplotypes were then constructed at the COL4A3/COL4A4 and COL4A5 loci for affected and unaffected family members. Families were too small for formal linkage studies, but the probabilities of hematuria segregating with haplotypes within individual families were calculated using simple statistics.

This project had the approval of the Human Research Ethics Committee of the Austin and Repatriation Medical Center, and all subjects provided signed informed consent.

## RESULTS

Twenty-two families of Northern European, Southern European, and Asian backgrounds were studied (Fig. 1 and Table 1). The index cases included 19 females who had a median age of 44 years (range of 6 to 88). Their median urinary RBC count was 173,000/mL (range 74,000 to 2,180,000/mL). Only one (5%) had proteinuria  $>300$  mg/L, and all had normal serum creatinine levels.

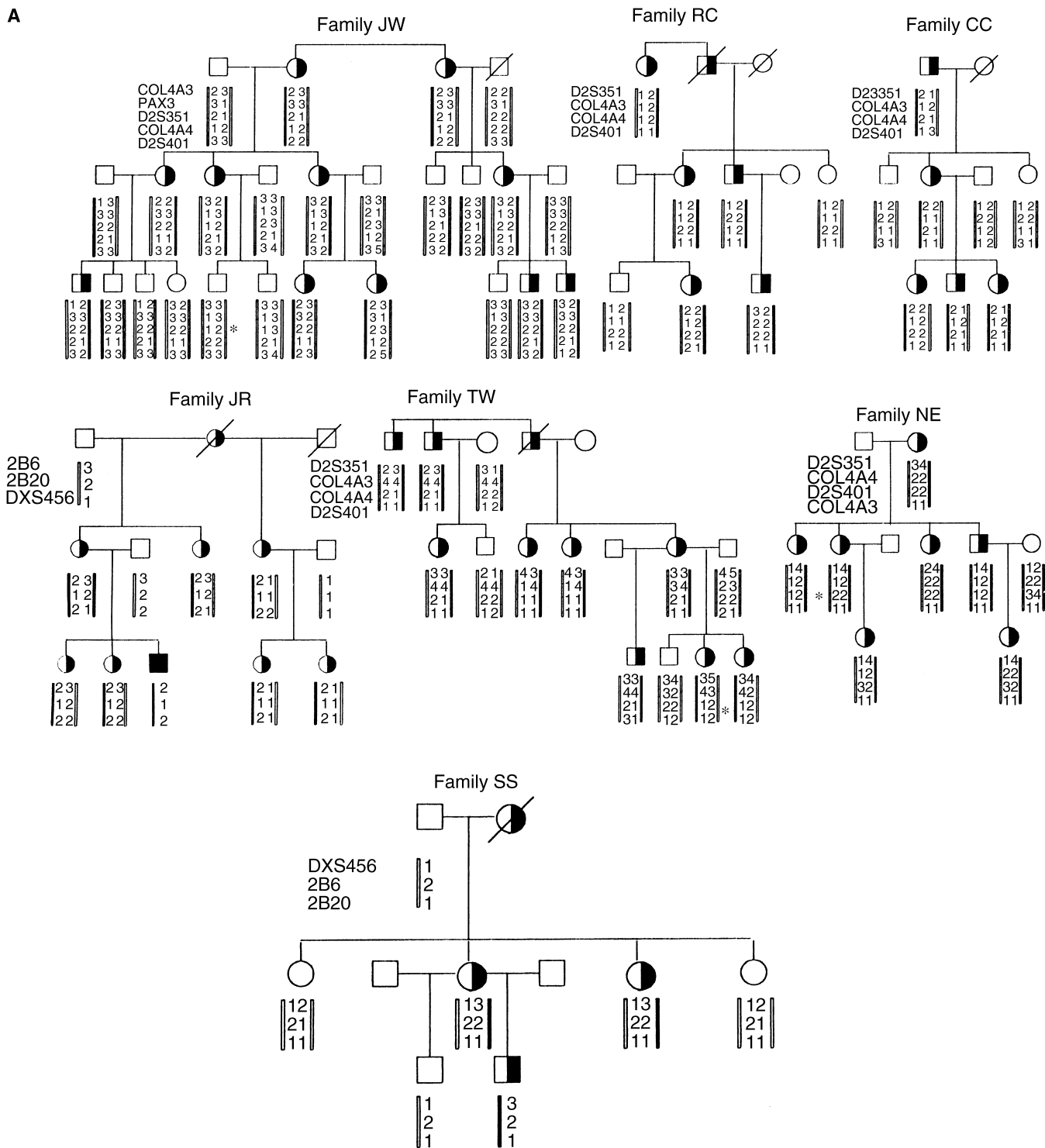
One hundred fifty-nine family members were studied, including 130 first-degree relatives and 29 unrelated spouses. The presence or absence of hematuria was confirmed in a second specimen in 87 cases, and in only one person were the findings inconsistent (the initial specimen had hematuria, but the second and third specimens were clear). Hematuria was present in 89 first-degree relatives (68%) and in 4 of the 29 (14%) unrelated spouses and was glomerular in all cases. Hematuria was demonstrated for the first time in 46 people (29% of everyone tested). It was more common in females (46 of 63, 73%) than in males (21 of 45, 47%) after the index cases had been excluded ( $P < 0.01$ ).

The families of 18 index cases had hematuria in at least two members. Ten families were aware of a positive family history before entering the study. Thus, 8 of the 12 families (67%) without a family history of TBMD actually had at least two members with hematuria.

Haplotypes were informative in all families, and the probability of hematuria segregating with a haplotype at a candidate locus in each family was calculated. For example, in Family MS (Fig. 1B), the putative hematuria haplotype at the COL4A3/COL4A4 locus was identified from the mother and daughter as 1211, and the likelihood of the elder son who is affected having this haplotype by chance was one half, and of the younger unaffected son not having this haplotype was also one half. Thus, the probability of hematuria in this family segregating with the 1211 haplotype was 0.25.

In 12 families, hematuria segregated with one of the candidate gene loci but not with the other (Table 1). Seven of these families were large enough that the results were significant. Hematuria segregated with the COL4A3/COL4A4 locus in five (all  $P < 0.05$ ) and was consistent with segregation only at this locus in a further three families. Hematuria segregated with the COL4A5 locus in two families ( $P < 0.05$ ) and was consistent with segregation only at this locus in a further two families.

In the 10 remaining families (45%), hematuria did not segregate with either gene locus even though some of these families were small, and four had only one person with hematuria. In order to identify further families in which incomplete penetrance of hematuria prevented segregation with a candidate locus, haplotypes of only the family members with hematuria were examined. However, this demonstrated no further families in which hematuria segregated with or was consistent with segregation at either locus.



**Fig. 1. (A) Families showing segregation of hematuria with haplotypes at COL4A3/COL4A4 (JW, RC, CC, TW, and NE) and COL4A5 (JR and SS) locus (all  $P < 0.05$ ).** In all of these families, hematuria segregated with only one of the two candidate gene loci. Families JR and SS show segregation of hematuria with haplotypes at COL4A5 ( $P < 0.05$ ). **(B) Families showing segregation of hematuria with haplotypes at COL4A3/COL4A4 (AC, JK, and MS) and COL4A5 (AV, SL) locus (all  $P = NS$ ).** In all of these families, hematuria segregated with only one of the two candidate gene loci. A new mutation was assumed in family SL in the index case who was the mother of the two girls. Hematuria did not segregate with haplotypes at either locus in the remaining 10 families. The asterisk indicates recombination.

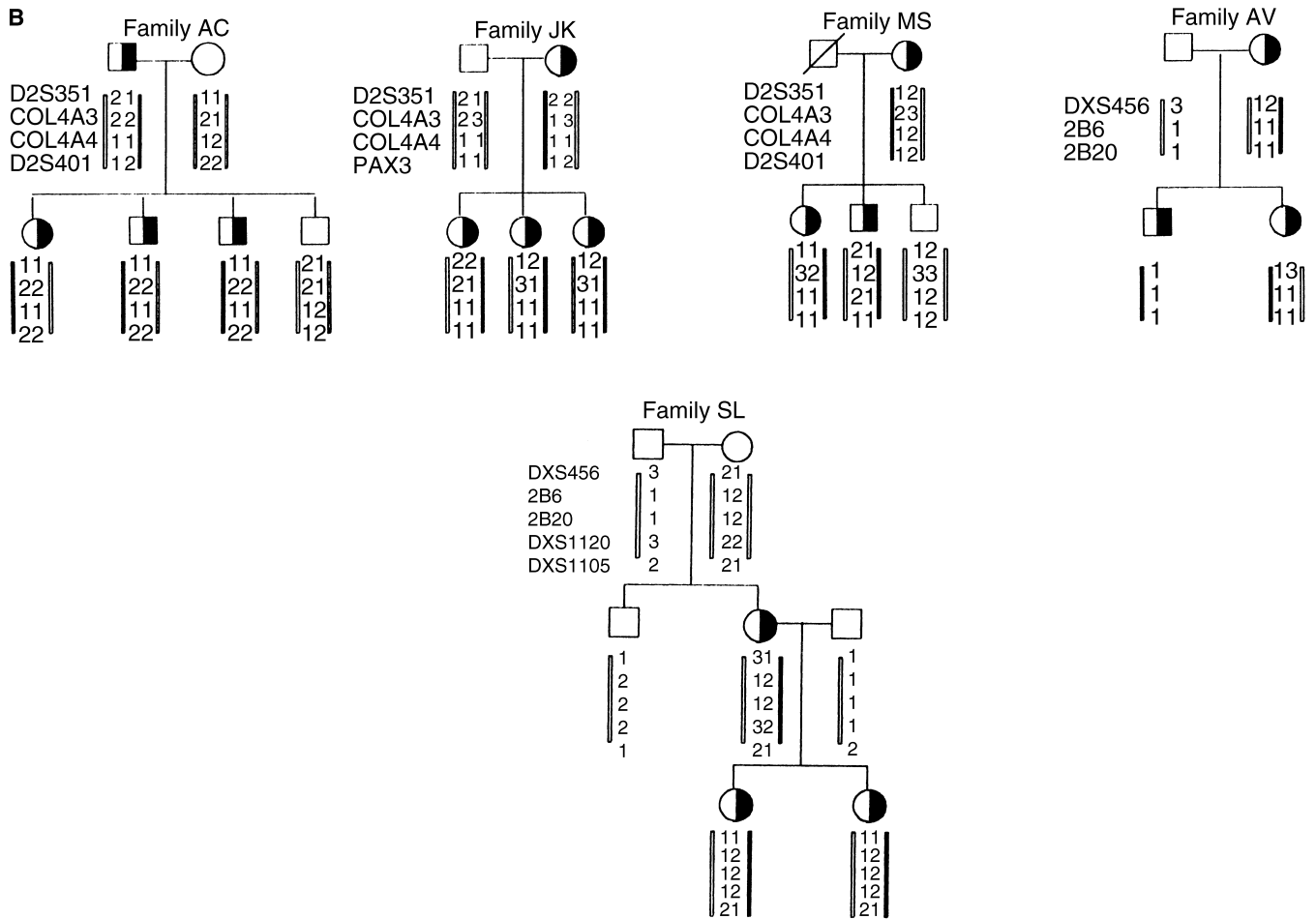


Fig. 1. (Continued)

Of the four families in which hematuria segregated with or was consistent with segregation at the COL4A5 locus, the absence of males with renal failure, hearing loss, and eye abnormalities was verified in two families (SL and AV). However, these families were small and did not contain any adult males, and the disease may have been due to recent mutations. In the other two families, the diagnosis of X-linked Alport syndrome was suspected during the course of the study in individuals who were not initially tested. In one family, the index case's six-year-old son was found to be deaf, and a subsequent renal biopsy demonstrated GBM thinning and marked lamellation. In the other family, a 55-year-old great uncle was admitted to the hospital with an acute myocardial infarct and died the next day. He did not have diabetes. His urine was positive for blood on dipstick and his serum creatinine was 0.55 mmol/L, but he was not deaf and had no known eye abnormalities. His mother and three sisters had hematuria, but neither his blood nor tissue was kept for DNA studies. He had no children.

An affected individual aged 28 years in a family (JW)

in which hematuria segregated with the COL4A3/COL4A4 locus had a serum creatinine of 0.28 mmol/L, and a renal biopsy showed TBMD and strong mesangial IgA immunofluorescence. Three of the 13 glomeruli were partly or completely sclerosed, and there was moderate interstitial fibrosis with areas of prominent tubular atrophy and minimal vascular changes. His serum creatinine four years previously had been 0.24 mmol/L.

Hematuria was confirmed in the four unrelated spouses. This had been recognized previously in two who had hypertension and "glomerulonephritis." None had had a renal biopsy; however, both parents of the third tested negative for hematuria, and the fourth had no parents or sibs alive who could be tested. Three of these individuals had married into families in which hematuria segregated with COL4A3/COL4A4. None of the four offspring who had inherited the hematuria haplotype from the other parent had renal failure or the hearing loss or eye manifestations typical of autosomal recessive Alport syndrome.

**Table 1.** Characteristics of proband, family members and results of linkage studies

Proband (gender, age)	Urinary RBC (10 <sup>3</sup> /mL) in proband	N family members tested	N first degree relatives tested	N first degree relatives with hematuria	COL4A5	COL4A3/ COL4A4	P
JR (F, 6)	1,600	11	8 (1M, 7F)	8 (1M, 7F)	Yes	No	0.016
JW (F, 36)	105	24	19 (10M, 9F)	11 (3M, 8F)	No	Yes	<0.0001
TW (M, 56)	120	13	11 (5M, 6F)	9 (3M, 6F)	No	Yes	0.00045
RC (F, 38)	240	7	7 (3M, 4F)	5 (2M, 3F)	No	Yes	0.008
CC (F, 9)	1,200	8	7 (2M, 5F)	5 (2M, 3F)	No	Yes	0.031
NE (F, 7)	340	8	7 (1M, 6F)	7 (1M, 6F)	No	Yes	0.016
SS (F, 38)	100	7	6 (2M, 4F)	3 (1M, 2F)	Yes	No	0.031
SL (F, 35)	950	7	3 (3F)	3 (3F)	Yes	No	0.50
AV (F, 10)	155	4	3 (1M, 2F)	3 (1M, 2F)	Yes	No	0.50
AC (M, 25)	410	6	5 (4M, 1F)	4 (3M, 1F)	No	Yes	0.125
JK (F, 57)	74	5	4 (4F)	4 (4F)	No	Yes	0.25
MS (F, 68)	240	4	4 (2M, 2F)	3 (1M, 2F)	No	Yes	0.25
MC (F, 49)	2,180	7	6 (3M, 3F)	4 (2M, 2F)	No	No	NS
NM (F, 52)	180	7	6 (3M, 3F)	2 (1M, 1F)	No	No	NS
RB (F, 32)	130	6	5 (2M, 3F)	2 (2F)	No	No	NS
MB (F, 64)	260	7	6 (3M, 3F)	1 (1F)	No	No	NS
HC (F, 55)	110	5	4 (1M, 3F)	3 (3F)	No	No	NS
MK (M, 58)	435	5	4 (2M, 2F)	3 (2M, 1F)	No	No	NS
BS (F, 68)	90	5	4 (1M, 3F)	4 (1M, 3F)	No	No	NS
JO (F, 59)	165	5	4 (4F)	3 (3F)	No	No	NS
MW (F, 39)	730	4	4 (1M, 3F)	1 (1F)	No	No	NS
IC (F, 60)	100	4	3 (1M, 2F)	1 (1F)	No	No	NS
Total 3M, 19F	74–2,180	159	130 (48M, 82F)	89 (24M, 65F)			

Red blood cell (RBC) counts were demonstrated by phase contrast microscopy. Number (N) family members tested include unrelated spouses. Abbreviations are: M, male; F, female. Number (N) first degree relatives tested may have included a selection bias, since affected members were more likely to offer to be tested. COL4A5 and COL4A3/COL4A4 refer to whether haplotypes at these loci segregated with hematuria in families of probands. Hematuria segregated with one of these loci in 7 families, was consistent with segregation at one of these loci in a further 5, and did not segregate at either locus in 10 families. In no family did hematuria segregate with both loci simultaneously.

## DISCUSSION

This study demonstrated that hematuria in families with TBMD commonly segregates with the COL4A3/COL4A4 locus and thus results from mutations in the same genes as autosomal recessive Alport syndrome. Furthermore, TBMD may sometimes be confused with the carrier state for X-linked Alport syndrome. However, nearly half the families in this study had hematuria that did not segregate with the loci for either autosomal recessive or X-linked Alport disease.

All index cases had thinning of the GBM on renal biopsy. At the start of the study, none had any clinical features or knew of any family members of any age with clinical features that suggested X-linked Alport syndrome, and none had extensive lamellation of the GBM that might also have been consistent with this diagnosis. The four index cases subsequently shown to have X-linked disease were all female, included one child, and had predominantly GBM thinning. It should be emphasized that there are no ultrastructural criteria that will distinguish between carriers of X-linked and autosomal recessive Alport syndrome.

Glomerular hematuria was used to identify affected individuals within families, because it is the most common feature of TBMD and persists throughout life [15]. In contrast, proteinuria is uncommon and renal function

is usually normal. The phase-contrast microscopic demonstration of hematuria represents the “gold standard” and is highly sensitive, specific, and reproducible in our laboratory. All hematuria demonstrated in this study in both family members and unrelated spouses contained at least three populations of RBC and was thus dysmorphic or “glomerular” in origin.

In this study, index cases were predominantly female, and female family members had hematuria more often than males. This may be partly because of a selection bias or because TBMD affects or is recognized more often in females than in males. At least one other clinical study has also found TBMD more often in females [16]. The female GBM is normally thinner than in males [17], and a genetic mutation that causes further attenuation may increase the likelihood of hematuria. On the other hand, in some of our families, hematuria segregated with the locus for X-linked disease, but few males were available for testing. In such families, the initial recognition of a male with renal impairment would have suggested the diagnosis of X-linked Alport syndrome and precluded this family from examination in our study.

Hematuria was inherited in two thirds of our families with TBMD in which a family history had not been recognized previously. Other causes of inherited hematuria are rare, and thus, even when there is no renal biopsy, the diagnosis of TBMD can usually be inferred

if the urine of the patient's parents, sibs, or children contains blood of glomerular origin [2]. Families with only one affected member may occur because of reduced penetrance of hematuria or de novo mutations or because families are small.

Efforts were made to recruit as many family members as possible, but members of families with several affected individuals were more likely to cooperate. The families were too small for formal linkage studies, and the significance of the results depended both on the strict segregation of hematuria with a haplotype at the candidate gene locus and also with the number of family members examined. This study represents an unselected unbiased series of consecutive families and patients with TBMD associated with our medical center rather than a study of only families large enough to produce significant results. It reflects the type of patients who present with hematuria for nephrological consultation. Although some families had fewer than seven first-degree relatives (usually the critical number to demonstrate significance), their small size still allowed us to exclude both candidate gene loci in 10 cases. Four of these families had only one affected individual, suggesting low penetrance of hematuria and that testing further members might not increase the significance of the results.

In 12 families, hematuria segregated with haplotypes at either the COL4A3/COL4A4 or COL4A5 locus, but not at both loci simultaneously. These results were significant, however, in only seven families, with hematuria segregating with the COL4A3/COL4A4 locus in five families and with the COL4A5 locus in two. Hematuria was consistent with segregation at the COL4A3/COL4A4 locus in a further three families and at the COL4A5 locus in the remaining two families. While the results in these five families were not significant, the lack of segregation at the other candidate locus increases the likelihood of these observations not occurring by chance alone. Thus, TBMD is more often due to mutations in the COL4A3 and COL4A4 genes than in the COL4A5 gene.

It is uncertain how often individuals from families in which hematuria segregates with the COL4A3/COL4A4 locus are actually carriers for autosomal recessive Alport syndrome. If most cases of autosomal recessive Alport syndrome resulted from the mating of two unrelated individuals with TBMD, then the prevalence of "compound heterozygotes" with autosomal recessive Alport syndrome should be approximately  $1/4 \times 1/100 \times 1/100$  (for the minimal prevalence of TBMD), which it approximates [18]. TBMD and Alport syndrome would then be analogous to  $\beta$  thalassemia trait, which causes only mild anemia, and thalassemia major, which results in severe disease, respectively. On the other hand, individuals with TBMD might just have autosomal dominantly inherited hematuria, and "compound heterozygotes" might infrequently have the clinical features of autosomal recessive

Alport syndrome. We were unable to demonstrate that any of the four offspring who had inherited the hematuria haplotype from one parent and had another parent with hematuria also had renal failure, deafness, or the eye abnormalities typical of autosomal recessive disease. However, none of the unrelated spouses with hematuria had had a renal biopsy-confirmed diagnosis of TBMD.

In two of the four families in which hematuria segregated with the locus for X-linked Alport syndrome, males with a hearing loss or hematuria came to our attention after the families had been studied. However, there were no males with renal failure or hearing loss in the other two families with probable X-linked disease. Thus, even a careful family history in a female carrier of X-linked Alport syndrome may not indicate this form of inheritance, especially where there are few males in the family, the only males are children, where the proband has a de novo mutation, or where the clinical manifestations of Alport syndrome develop in later life.

Families in which hematuria did not segregate with a haplotype at the candidate gene loci for TBMD may have occurred because of reduced penetrance of hematuria [8, 15]. Hematuria probably occurs in most carriers of X-linked Alport syndrome, but in only approximately 80% of carriers of autosomal recessive disease [8]. In order to overcome the effect of incomplete penetrance of hematuria, pedigrees were re-examined taking into account only individuals with hematuria, but no further families in which hematuria segregated with haplotypes at the candidate gene loci were identified. However, our ability to demonstrate segregation of hematuria with haplotypes at the COL4A3/COL4A4 locus at all suggests that hematuria is fully penetrant in at least some families, and possibly depends on the nature of the underlying genetic mutations.

Another explanation for the lack of segregation of hematuria with haplotypes at candidate gene loci is the presence of de novo mutations. These occur in approximately 15% of individuals with X-linked Alport syndrome [19], but their frequency in the COL4A3 and COL4A4 genes is not known.

A novel gene locus for TBMD could also account for our inability to demonstrate segregation of hematuria with the candidate gene loci in all families. If TBMD represents a carrier state for autosomal recessive Alport syndrome, and autosomal recessive and dominant conditions often affect the same gene, then the new locus for autosomal dominant disease [20] may also represent a locus for TBMD.

Finally, in some families in which hematuria did not segregate with the candidate gene loci, this might have occurred because of coincidental hematuria from other causes in family members who did not have the "hematuria haplotype."

Most patients with TBMD have normal renal function, but occasionally, individuals with an elevated creatinine

are described. In our study, one such individual was demonstrated to have IgA glomerulonephritis in addition to TBMD on his renal biopsy. Many forms of glomerulonephritis, especially IgA nephropathy, are more common in patients with TBMD [21] and are associated with a worse outcome than occurs with TBMD alone [22].

Thus, hematuria in individuals with TBMD is more likely to segregate with the locus for autosomal recessive than for X-linked Alport syndrome. When individuals are heterozygous for mutations in the autosomal recessive Alport locus, members of subsequent generations will have hematuria, but serious kidney disease is unlikely, whereas some family members of carriers of X-linked disease are likely to develop renal failure. TBMD did not segregate with the locus for autosomal recessive or X-linked Alport syndrome in nearly half of all of the families we examined. The reasons for this are unclear.

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